

A Robust Noninvasive Approach to Study Gut Microbiota Structure of Amphibian Tadpoles by Feces

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Abstract The 16S rDNA amplicon high-throughput sequencing technique provides a robust and inexpensive approach to detect the gut microbiota of amphibians. Since different experimental protocols generate technical biases in drawing the gut microbiota profiles, the integrative analysis of gut microbiota produced by different studies must be performed with circumspection. In this study, we compared the efficacy of two DNA extraction methods (i.e., a phenol-chloroform method and TIANamp Stool DNA Kit) in describing intestinal and fecal bacterial communities of transplanted Asiatic toad (*Bufo gargarizans*) tadpoles. In terms of the DNA extraction quality (i.e., DNA purity and yield rate) and the consistency in between fecal and intestinal microbiota structures (i.e., α and β diversity indices), the phenol-chloroform method was more robust than this commercial stool kit in profiling gut microbiota of tadpoles with feces.

Keywords *Bufo gargarizans*, DNA extraction, intestinal microflora, Phenol-chloroform, 16S rDNA

1. Introduction

Knowledge about the diversity and composition of gut microbiota has accumulated rapidly by culture-independent methods, especially the 16S rDNA amplicon high-throughput sequencing technique (Su *et al.*, 2012). However, different experimental protocols concerned with material preparation, DNA extraction and PCR primer selection can generate technical biases in drawing the gut microbiota profiles (Larsen *et al.*, 2015; Lozupone *et al.*, 2013; Wagner Mackenzie *et al.*, 2015).

Since acquiring high quality genomic DNA is the prerequisite for the downstream analysis of gut microbiota structure, researchers have performed many studies on the effect of material types, sample storage conditions and DNA extraction methods (Choo *et al.*, 2015; Ferrand *et al.*, 2014; Larsen *et al.*, 2015; Song *et al.*, 2016;

Wagner Mackenzie *et al.*, 2015). In spite of differences between intestinal and fecal microflora (Eckburg *et al.*, 2005; Nava *et al.*, 2011), feces have been utilized as a noninvasive material to measure the intra- and inter-individual variation in composition and diversity of intestinal bacterial communities (Arumugam *et al.*, 2011; Dethlefsen *et al.*, 2008; Yatsunenko *et al.*, 2012). Storage conditions for fecal samples could significantly influence microbiota profiles (Bahl *et al.*, 2012; Choo *et al.*, 2015; Maukonen *et al.*, 2012), though some exceptions exist (Fouhy *et al.*, 2015). DNA extraction protocols can produce technical variations due to different cell lysis and purification methods (Claassen *et al.*, 2013; Maukonen *et al.*, 2012; Wagner Mackenzie *et al.*, 2015; Yuan *et al.*, 2012). However, these technical variations seem not to be large enough to distort the biological variations (Lozupone *et al.*, 2013; Wagner Mackenzie *et al.*, 2015).

Recent research on gut microbiota has shed new light on the associations between gut microbiota and vertebrate physiology, development and evolution (Ley *et al.*, 2008; McFall-Ngai *et al.*, 2013; Nicholson *et al.*, 2012; Yatsunenko *et al.*, 2012), while the number of intestinal microflora studies in amphibians remains far smaller than that in mammals (Bletz *et al.*, 2016; Colombo *et al.*, 2015;

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Jiménez and Sommer, 2017; Kohl *et al.*, 2013; Kohl *et al.*, 2014; Mashoof *et al.*, 2013; Vences *et al.*, 2016; Weng *et al.*, 2016; Weng *et al.*, 2017). Intensive studies of the gut microbiota in different taxa including amphibians are essential prerequisites to elucidate host-gut microbiota symbioses, e.g., phylosymbiosis (Brooks *et al.*, 2016; Ley *et al.*, 2008; Li *et al.*, 2017a; Li *et al.*, 2017b; Shapira, 2016; Vences *et al.*, 2016). In addition, the understanding of gut microbiota in amphibians could help us to take effective measures for amphibian conservation and cultivation (Jiménez and Sommer, 2017). Since many amphibians have been suffering from severe survival conditions (Hof *et al.*, 2011), noninvasive approaches will always be an optimal choice for the study of symbioses between amphibians and gut microbiota. However, it remains unclear whether the feces can be applied as a noninvasive material to study the gut microbiota of amphibians.

In this study, we aimed to test the efficacy of a phenol-chloroform method and a commercial fecal reagent kit (TIANGEN Biotech Co., Ltd.) in describing intestinal bacterial communities of Asiatic toad (*B. gargarizans*) tadpoles by feces. Specifically, DNA extraction quality of different methods and sample types was tested in terms of three parameters, i.e., A260/A280, A260/A230 and DNA yield rate. Furthermore, the structural consistency between bacterial communities was evaluated based on 16S rDNA amplicon high-throughput sequencing.

2. Materials and Methods

2.1. Transplantation of *B. gargarizans* eggs In February-March 2016, we sampled seven broods of *B. gargarizans* eggs from Xinyang City of Henan Province in China (Table S1). A ~10 cm-length chalaza was taken from each brood of eggs, and then was hatched in labs using plastic cylinders (1 L) filled with about 0.5 L dechlorinated drinking water. Subsequently, about 15 larvae in each brood were reared together with boiled green vegetable leaves rich in cellulose or fish foods rich in protein. We applied semi-natural conditions for rearing all larvae, i.e., a water change and food feeding per three days without controlling the light, humidity and temperature. All procedures used in this study were approved by the Animal Care and Use Committee of Xinyang Normal University.

2.2. Preparation of intestinal and stool samples When tadpoles developed into the lower limb stage (Gosner 35–40), we collected intestinal and stool samples. We first collected 10 samples (S1–S10) of mixed fecal sediments

from cultivation water by using aseptic injectors (Table S1). These mixed stool samples spin-dried in a centrifuge were applied for comparing DNA extraction quality of the phenol-chloroform method and the TIANamp Stool DNA Kit. In addition to the DNA extraction quality, 23 tadpoles (S11–S33) were further selected to compare the microbiota structure between the phenol-chloroform method and the commercial stool kit (Table S1). Tadpoles (S11–S16 and S22–S27) were individually cultivated for less than 24 hours after a water change. Then we got their spin-dried stool samples through the same approach for mixed stool samples. We sacrificed tadpoles using 75% ethanol solution before the extraction of the gut samples into sterile microcentrifuge tubes. All intestinal and stool samples were stored in a –20°C freezer before the DNA extraction.

2.3. Protocols of DNA extraction and DNA quality evaluation Phenol-chloroform method.—The 500 µL SDS (1%) and each weighed sample were mixed in a sterile 1.5 mL microtube and bathed in the water for 10 min at 60°C. During the water bath, microtubes were overturned and blended for three times. Subsequently, 30 µL EDTA (0.5 M) and 20 µL protease K (20 mg/mL) were added into each microtube, and the mixture was bathed in the water for 1 hour at the same temperature of the previous step. And then the samples were centrifuged at 12 000 rpm for 5 min at 4°C. The supernatant fraction was transferred to a new 1.5 mL microtube, and blended with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 in volume). The samples were centrifuged at 12000 rpm for 5 min at 4°C once again. The supernatant fraction was transferred to a new 1.5 mL microtube, and blended with an equal volume of chloroform-isoamyl alcohol (24:1 in volume). The samples were centrifuged at 12000 rpm for 5 min at 4°C once again. The supernatant fraction was transferred to a new 1.5 mL microtube, and blended with twofold absolute ethanol. After cooled at –20°C for 20 min, the samples were centrifuged at 12 000 rpm for 10 min at 4°C. After the removal of the supernatant fractions, the DNA precipitates were washed in 200 µL ethanol (70%) for 5 min, and then the mixtures were centrifuged at 12 000 rpm for 5 min at 4°C. This step was repeated three times. The dry DNA was dissolved in 40 µL TE buffer solution.

TIANamp stool DNA kit.—TIANamp Stool DNA Kit simplifies the DNA isolation by a fast spincolumn procedure. The protocol recommended by the manufacturer was utilized to extract the metagenomic DNA.

DNA quality evaluation.—The A260/A280, A260/A230 and DNA yield rate were applied for the DNA quality evaluation. The A260/A280, A260/A230 and concentration of DNA products were determined by using NanoVue Plus Spectrophotometer (GE Healthcare Inc., Germany). The DNA yield rate was given by the ratio of the DNA concentration to the sample weight. The DNA products extracted from feces or intestines of tadpoles (S11–S16 and S22–S27) were subsequently stored in a –20°C freezer until the high-throughput sequencing of 16S rDNA amplicons.

2.4. 16S rDNA amplicon high-throughput sequencing
 The library construction of 16S rDNA amplicons and high-throughput sequencing on MiSeq (Illumina Inc., USA) were achieved in a commercial company (Genergy Inc., China). Specifically, the hypervariable regions of V3–V4 in the bacterial 16S rDNA were amplified from the microbiota DNA products using the universal primer pair 341F–CCTACGGGNGGCWGCAG and 785R–GACTACHVGGGTATCTAATCC (Klindworth *et al.*, 2013). The amplicons were generated by a two-step, tailed PCR on the DNA products in terms of the 16S Metagenomic Sequencing Library Preparation protocol with some modifications (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). The volume of each PCR solution was 25 μL, which consisted of 10 ng DNA, 2.5 μL 10 × Takara Ex Taq Buffer, dNTP (2.5 mmol/L each), 5 pmol/L forward primer, 5 pmol/L reverse primer, 0.1 μL Takara Ex Taq, and ddH₂O. The 1st PCR condition was 94°C for 3 min, 20 cycles (94°C for 10 s, 55°C for 15 s, 72°C for 30 s) and 72°C for 7 min. The 2nd PCR condition was 94°C for 3 min, 5 cycles (94°C for 10 s, 55°C for 15 s, 72°C for 30 s) and 72°C for 7 min. Finally, the 16S rDNA amplicons quantified by Quant-iTTM PicoGreen[®] dsDNA Assay Kit (Life Technologies, USA) were paired-end sequenced on the Illumina MiSeq platform.

2.5. Bioinformatic analyses of 16S rDNA amplicons
 The raw paired-end reads, after the removal of barcodes, were filtered by Trimmomatic software with three minimum thresholds (Bolger *et al.*, 2014), i.e., terminal base quality score equal to 25, average quality score equal to 25 in sliding windows of 50 bp with a step of 1 bp, fragment length equal to 100 bp. The paired-end sequences were merged with the flash software for a minimum overlap of 10 bp and a maximum mismatch proportion of 0.2. The merged sequences including ambiguous bases were excluded. Subsequently, the 16S rDNA reference database downloaded from NCBI was

utilized for the validation of V3–V4 regions. In this study, 16S rDNA amplicons were successfully sequenced in 32 samples excluding S14_S, S17_G and S20_G (Tables 1, S1).

Table 1 Sequencing results for successfully sequenced 32 samples.

Parameter	Value
Number of total sequences	811 311
Number of total bases	3.74×10^8
Minimum sequence length	124
Maximum sequence length	510
Mean of sequence lengths	460.71
Median of sequence lengths	465
Standard deviation	11.81
GC percentage	0.5384
N50	465

Operational taxonomic units (OTUs) were assigned to the 16S rDNA amplicons using UPARSE software with the identity threshold of 97% (Edgar, 2013). The annotation of representative OTU sequences was executed through sequence alignments on the RDP database using the RDP classifier (confidence threshold = 0.8) (Wang *et al.*, 2007).

The mothur program was used to calculate α diversity indexes (Schloss *et al.*, 2009), i.e., richness (OTU observed, ACE and Chao1), diversity and evenness (Shannon, Simpson and Shannoneven), and Good's coverage. The rarefaction and Shannon curves were drawn to measure whether the sequencing depth was appropriate for the richness and diversity calculation. To statistically analyze the differences in α and β diversity indexes between samples, 16 881 (i.e., minimum number of sequences in all samples) sequences in each sample were subsampled. In addition, we drew stackbars and Venn charts to show the taxonomic composition and abundance of samples. Furthermore, we used Past software (v3.14) to execute principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity calculated from taxonomic abundances to analyze the β diversity of microbiota structure (Hammer *et al.*, 2001). The transformation exponent was set to the default value (i.e., 2).

2.6. Statistical analyses of the DNA extraction quality and the microbiota structure To compare the DNA extraction quality of phenol-chloroform method and the TIANamp Stool DNA Kit, we utilized SPSS software (v20.0, IBM Corporation) to perform Mann-Whitney

Rank Sum Test and two-way ANOVA (two factors: experimental material and DNA extraction method) in terms of A260/A280, A260/A230 and DNA yield rate. Pairwise multiple comparisons were performed in two-way ANOVA with Bonferroni correction when overall significance level was less than 0.05. In addition, fitting curves, which sketched the relationship of the DNA yield rate and sample weight, were compared between the phenol-chloroform method and the commercial stool kit.

To test the effect of experimental material and DNA extraction method on the microbiota structures, we executed two-way ANOVA to compare α diversity indexes of intestinal and fecal microbiota in S11–S33 tadpoles. We hypothesized that the factors, i.e., host genetic background and diet, could bias the comparative analysis of microbiota structure. Therefore, we used paired *t*-Test or Wilcoxon Signed Rank Test to compare α diversity indexes of gut microbiota between 10 pairs of littermate tadpoles (S11–S16, S18–S21 and S24–S33), i.e., S11_G–S16_G, S18_G–S21_G versus S24_G–S33_G (*Gut_ph* vs *Gut_kit*). Subsequently, the comparisons of α diversity indexes between intestinal and fecal microbiota were performed on 12 tadpoles (S11–S16 and S22–S27), i.e., S11_S–S16_S versus S11_G–S16_G (*Stool_ph* vs *Gut_ph*) and S22_S–S27_S versus S22_G–S27_G (*Stool_kit* vs *Gut_kit*). The intestinal/fecal metagenomic DNA of S11–S16 and S22–S27 was extracted using the phenol-chloroform method and TIANamp Stool DNA Kit,

respectively.

In addition to permutational multivariate ANOVA (PERMANOVA), we also performed a Mantel test on the Bray-Curtis dissimilarities calculated from taxonomic (i.e., OTU, genus and phylum) abundances in the above three cases, i.e., *Gut_ph* vs *Gut_kit*, *Stool_ph* vs *Gut_ph* and *Stool_kit* vs *Gut_kit*. The number of permutations was set to 9999 for PERMANOVA and Mantel tests. To test which taxonomies significantly affected the structural divergence of microbiota in these three cases, Lda Effective Size (LEfSe) were executed with recommended options (<http://huttenhower.sph.harvard.edu/galaxy/>).

3. Results

3.1. Efficacy comparison in terms of DNA purity and yield rate Among the tests of the between-method difference in A260/A280, A260/A230 and DNA yield rate, a significant difference was detected in A260/A230 (Mann-Whitney Rank Sum Test: $U = 205$, $n_1 = 23$, $n_2 = 27$, $P = 0.04$) and DNA yield rate ($U = 66$, $n_1 = 15$, $n_2 = 17$, $P = 0.02$) but not A260/A280 ($U = 300.5$, $n_1 = 23$, $n_2 = 27$, $P = 0.85$) (Table S1; Figures 1, 2). Whereas when the two-way ANOVA was applied (Table 2), a significant between-method difference was detected in A260/A230 and DNA yield rate but not in A260/A280. A significant between-material difference was detected in A260/A280 and A260/A230 but not in DNA yield rate. No significant

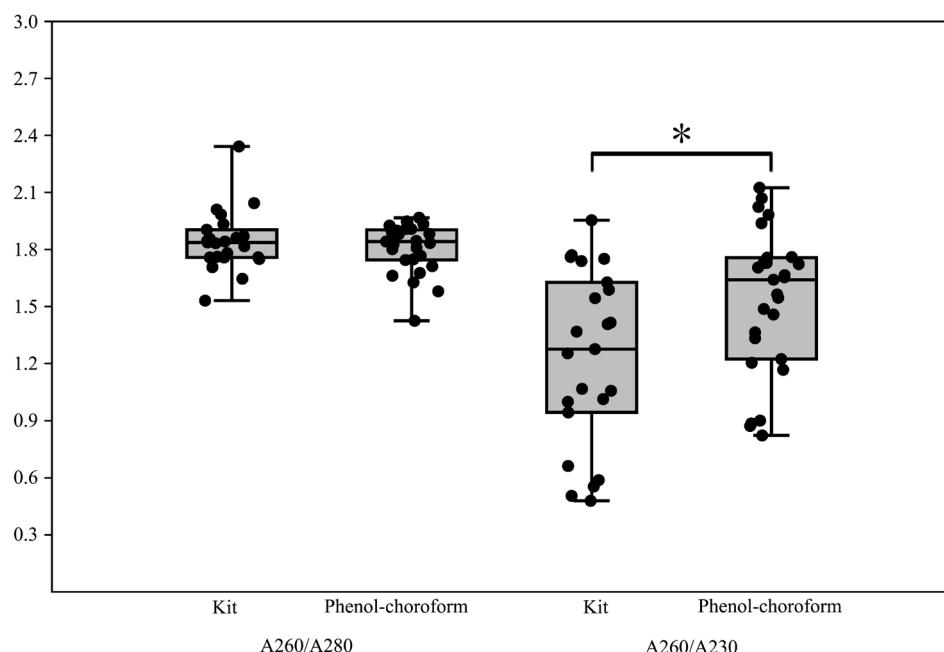


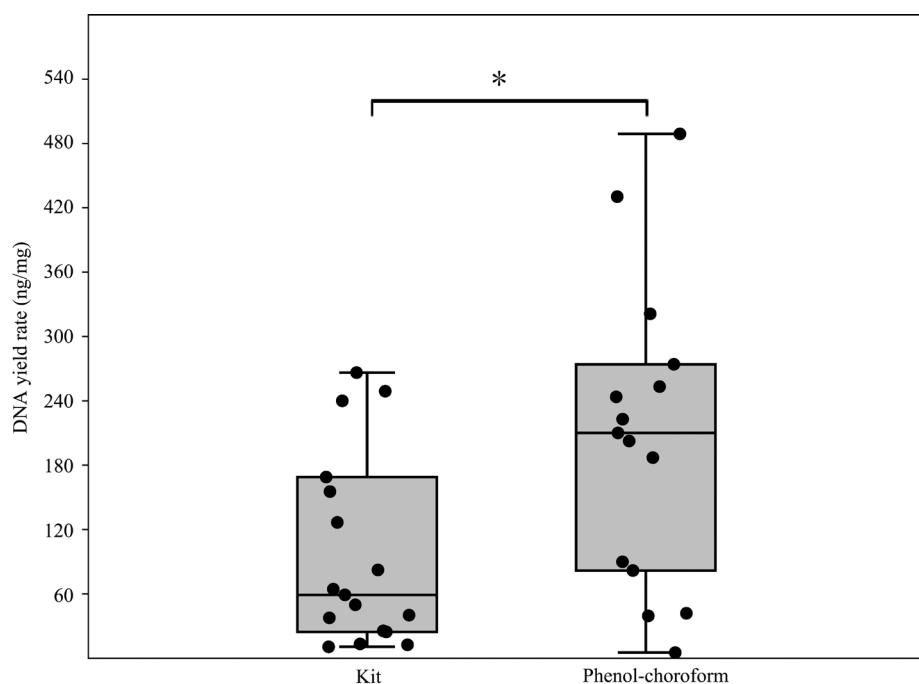
Figure 1 Comparisons of A260/A280 and A260/A230 between TIANamp Stool DNA Kit ($n = 23$) and phenol-chloroform method ($n = 27$).
* $P < 0.05$.

Table 2 Two-way ANOVA on A260/A280, A260/A230 and DNA yield rate.

Source of variation		Degree of freedom	Sum of squares	Mean square	F	P
A260/A280	Materials	2	0.14	0.07	3.73	0.03
	Methods	1	0.06	0.06	3.09	0.09
	Materials × Methods	2	0.07	0.03	1.83	0.17
A260/A230	Materials	2	1.94	0.97	7.22	< 0.01
	Methods	1	0.70	0.70	5.21	0.03
	Materials × Methods	2	0.63	0.31	2.33	0.11
DNA yield rate	Materials	2	48 055.72	24 027.86	1.85	0.18
	Methods	1	123 332.77	123 332.77	9.49	< 0.01
	Materials × Methods	1	5490.65	5490.65	0.42	0.52

interaction was detected between these two factors (i.e., experimental material and DNA extraction method). In the pairwise multiple comparisons, A260/A230 (avg. \pm std. err.) of stool samples (1.05 ± 0.11 , $n = 12$) was significantly different from that of gut samples (1.51 ± 0.08 , $n = 23$) and mixed stool samples (1.51 ± 0.10 , $n = 15$). Furthermore, the fitting curves for DNA yield rate and sample weight show that the DNA yield rate in the phenol-chloroform method and the commercial stool kit decreases along with saturated DNA extraction materials in solutions (Figure S1). The phenol-chloroform method has a greater DNA yield rate than the commercial stool kit does at a given sample weight.

3.2 Efficacy comparisons in terms of microbiota structures In *Gut_ph* vs *Gut_kit*, total number of OTUs was 121, among which 4 belonged to *Gut_ph* (3.3%), 4 belonged to *Gut_kit* (3.3%) and 113 belonged to between groups (93.4%) (Figure 3). In *Stool_ph* vs *Gut_ph*, total number of OTUs was 100, among which 6 belonged to *Stool_ph* (6%), 8 belonged to *Gut_ph* (8%) and 86 belonged to between groups (86%). In *Stool_kit* vs *Gut_kit*, total number of OTUs was 114, among which 9 belonged to *Stool_kit* (7.9%), 7 belonged to *Gut_kit* (6.1%) and 98 belonged to between groups (86%). The Venn chart indicates that the majority of OTUs could be extracted by using the phenol-chloroform method or the

**Figure 2** Comparison of DNA yield rate between TIANamp Stool DNA Kit ($n = 17$) and phenol-chloroform method ($n = 15$). $^*P < 0.05$.

commercial stool kit from intestines or stools.

No significant differences in α diversity indexes were detected in between- material groups and between-method groups by using two-way ANOVA (Table S2). When host genetic background and diet factors were taken into account, we detected no significant differences in almost all α diversity indices in *Gut_ph* vs *Gut_kit*, *Stool_ph* vs *Gut_ph* and *Stool_kit* and *Gut_kit* (Table 3; Figure 4). One exception was that the richness values (i.e., number of observed OTUs) showed a significant difference between *Stool_kit* and *Gut_kit* ($P = 0.02$, $n = 6$) (Table 3; Figure 4).

None of the Bray-Curtis dissimilarities calculated

from OTU, genus and phylum abundances showed a significant between-group difference in *Gut_ph* vs *Gut_kit* (Table 4; Figures 5, S2, S3). However, only the Bray-Curtis dissimilarity based on genus abundances gave rise to a weak between-group correlation ($R = 0.32$, $P = 0.04$). In the PERMANOVA for *Stool_ph* vs *Gut_ph* (Table 4; Figures 5, S2, S3), the OTU, genus and phylum abundances also showed an insignificant between-group variation in terms of Bray-Curtis dissimilarity. In addition, both Bray-Curtis dissimilarities calculated from OTU and genus abundances showed a significant between-group correlation, i.e., $R = 0.64$ ($P = 0.04$) and $R = 0.58$ ($P = 0.02$). As for the *Stool_kit* vs *Gut_kit* (Table 4; Figures 5,

Table 3 Alpha diversity indexes (avg. \pm std. dev.) calculated from OTU tables rarefied to 16 881 sequences per sample in three cases, i.e., *Gut_ph* vs *Gut_kit* ($n = 9$), *Stool_ph* vs *Gut_ph* ($n = 5$) and *Stool_kit* vs *Gut_kit* ($n = 6$).

	<i>Gut_ph</i> vs <i>Gut_kit</i>		<i>Stool_ph</i> vs <i>Gut_ph</i>		<i>Stool_kit</i> vs <i>Gut_kit</i>	
	<i>Gut_ph</i>	<i>Gut_kit</i>	<i>Stool_ph</i>	<i>Gut_ph</i>	<i>Stool_kit</i>	<i>Gut_kit</i>
Richness	62.33 \pm 11.56	60.67 \pm 9.45	63.60 \pm 4.72	58.20 \pm 12.28	69.17 \pm 8.86	58.17 \pm 12.83
Shannon	1.84 \pm 0.63	2.08 \pm 0.55	1.82 \pm 0.52	1.62 \pm 0.52	1.76 \pm 0.49	1.80 \pm 0.46
Chao1	75.00 \pm 12.73	70.14 \pm 10.95	71.40 \pm 5.49	69.02 \pm 11.62	78.44 \pm 11.48	68.03 \pm 15.12
ACE	78.52 \pm 14.58	71.90 \pm 11.26	73.02 \pm 3.91	72.65 \pm 16.75	79.81 \pm 11.95	66.78 \pm 12.90
Simpson	0.33 \pm 0.20	0.25 \pm 0.16	0.32 \pm 0.18	0.39 \pm 0.18	0.37 \pm 0.19	0.30 \pm 0.17
Coverage	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00
Shannoneven	0.44 \pm 0.14	0.50 \pm 0.12	0.44 \pm 0.12	0.40 \pm 0.11	0.41 \pm 0.11	0.44 \pm 0.10

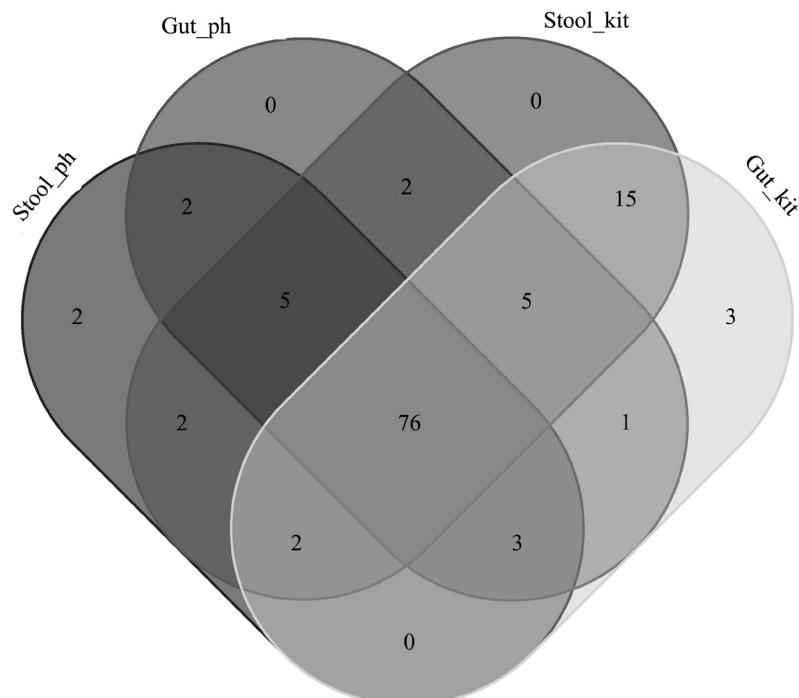


Figure 3 The distribution of OTUs in fecal and intestinal microbiota based on two DNA extraction methods.

Table 4 PERMANOVA and Mantel test on the Bray-Curtis dissimilarities calculated from taxonomic (i.e., OTU, genus and phylum) abundances in three cases, i.e., *Gut_ph* vs *Gut_kit* ($n = 9$), *Stool_ph* vs *Gut_ph* ($n = 5$) and *Stool_kit* vs *Gut_kit* ($n = 6$).

		<i>Gut_ph</i> vs <i>Gut_kit</i>			<i>Stool_ph</i> vs <i>Gut_ph</i>			<i>Stool_kit</i> vs <i>Gut_kit</i>		
		OTU	genus	phylum	OTU	genus	phylum	OTU	genus	phylum
PERMANOVA	Permutation No.	9 999	9 999	9 999	9 999	9 999	9 999	9 999	9 999	9 999
	<i>F</i>	1.25	1.02	0.50	0.63	0.44	0.58	4.33	4.37	2.51
	<i>P</i>	0.22	0.40	0.67	0.66	0.80	0.54	0.02	0.02	0.12
Mantel test	Permutation No.	9 999	9 999	9 999	9 999	9 999	9 999	9 999	9 999	9 999
	Correlation (<i>R</i>)	0.16	0.32	-0.06	0.64	0.58	0.28	0.65	0.64	-0.17
	<i>P</i>	0.21	0.04	0.54	0.04	0.02	0.22	0.01	0.01	0.75

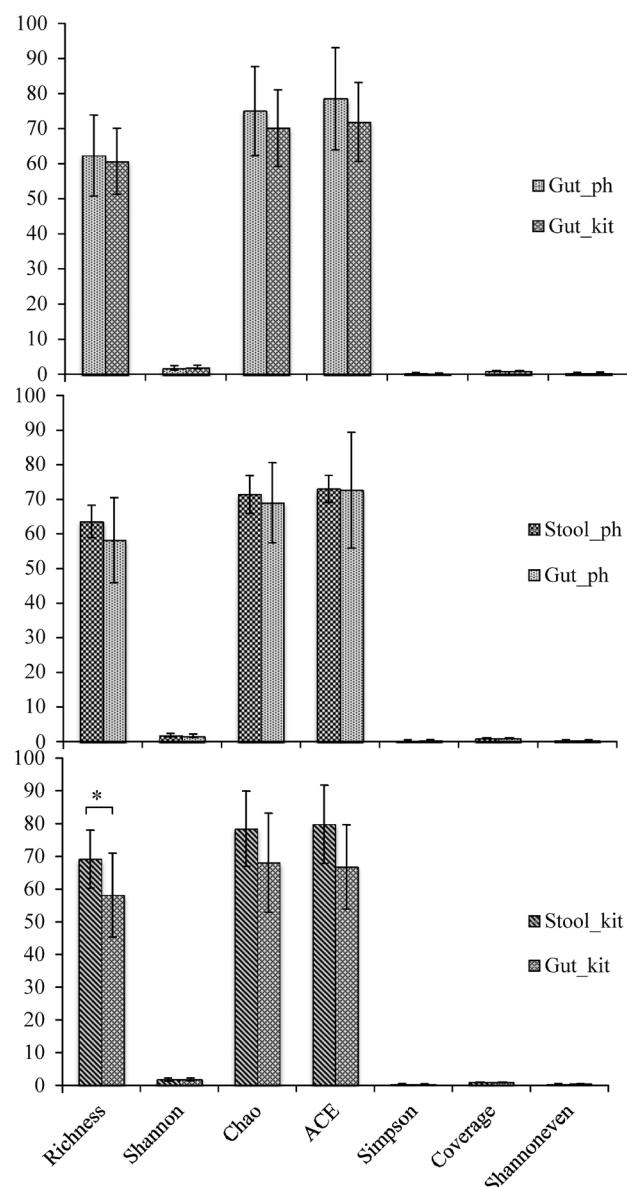


Figure 4 Comparisons of α diversity indices in three cases, i.e., *Gut_ph* vs *Gut_kit*, *Stool_ph* vs *Gut_ph* and *Stool_kit* vs *Gut_kit*. The bars and error bars represent mean values and standard deviations, respectively. $^*P < 0.05$.

S2, S3), both Bray-Curtis dissimilarities calculated from OTU and genus abundances also showed a significant between-group correlation, i.e., $R = 0.65$ ($P = 0.01$) and $R = 0.64$ ($P = 0.01$). Nevertheless, significant between-group variations was detected in both Bray-Curtis dissimilarities based on OTU and genus abundances (PERMANOVA: $F_{1,10} = 4.33$, $P = 0.02$) and $F_{1,10} = 4.37$, $P = 0.03$).

In *Gut_ph* vs *Gut_kit*, we identified by the LEfSe analysis that three genera of phylum Proteobacteria showed significant between-group divergence, i.e., *Sphingorhabdus* biased to *Gut_ph*, and *Coxiella* biased to *Gut_kit* along with an unnamed genus in order Rhizobiales (Figure 6). As for *Stool_ph* vs *Gut_ph*, five genera (i.e., *Hydrogenophaga*, *Rhizobium*, *Brevundimonas*, an unnamed genus in order Rhizobiales, an unnamed genus in family Sphingomonadaceae) in phylum Proteobacteria biased to *Stool_ph*, and genus *Clostridium_XIVa* in phylum Firmicutes biased to *Gut_ph* (Figure 6). In addition, significant between-group divergences of four phyla (i.e., Proteobacteria, Bacteroidetes, Verrucomicrobia, Firmicutes) composed of 15 genera were detected in *Stool_kit* vs *Gut_kit*, i.e., 12 genera in the phyla of Proteobacteria, Bacteroidetes and Verrucomicrobia biased to *Stool_kit*, and 3 genera in the phyla of Proteobacteria and Firmicutes biased to *Gut_kit* (Figure S4). The shared between-material bias of genera in *Stool_ph* vs *Gut_ph* and *Stool_kit* vs *Gut_kit* included four genera in phylum Proteobacteria biased to *Stool* (i.e., *Hydrogenophaga*, *Rhizobium*, *Brevundimonas*, an unnamed genus in family Sphingomonadaceae) and one genus in phylum Firmicutes biased to *Gut* (i.e., *Clostridium_XIVa*).

4. Discussion

The phenol-chloroform method is a classic and cost-effective approach to extract eukaryotic or prokaryotic

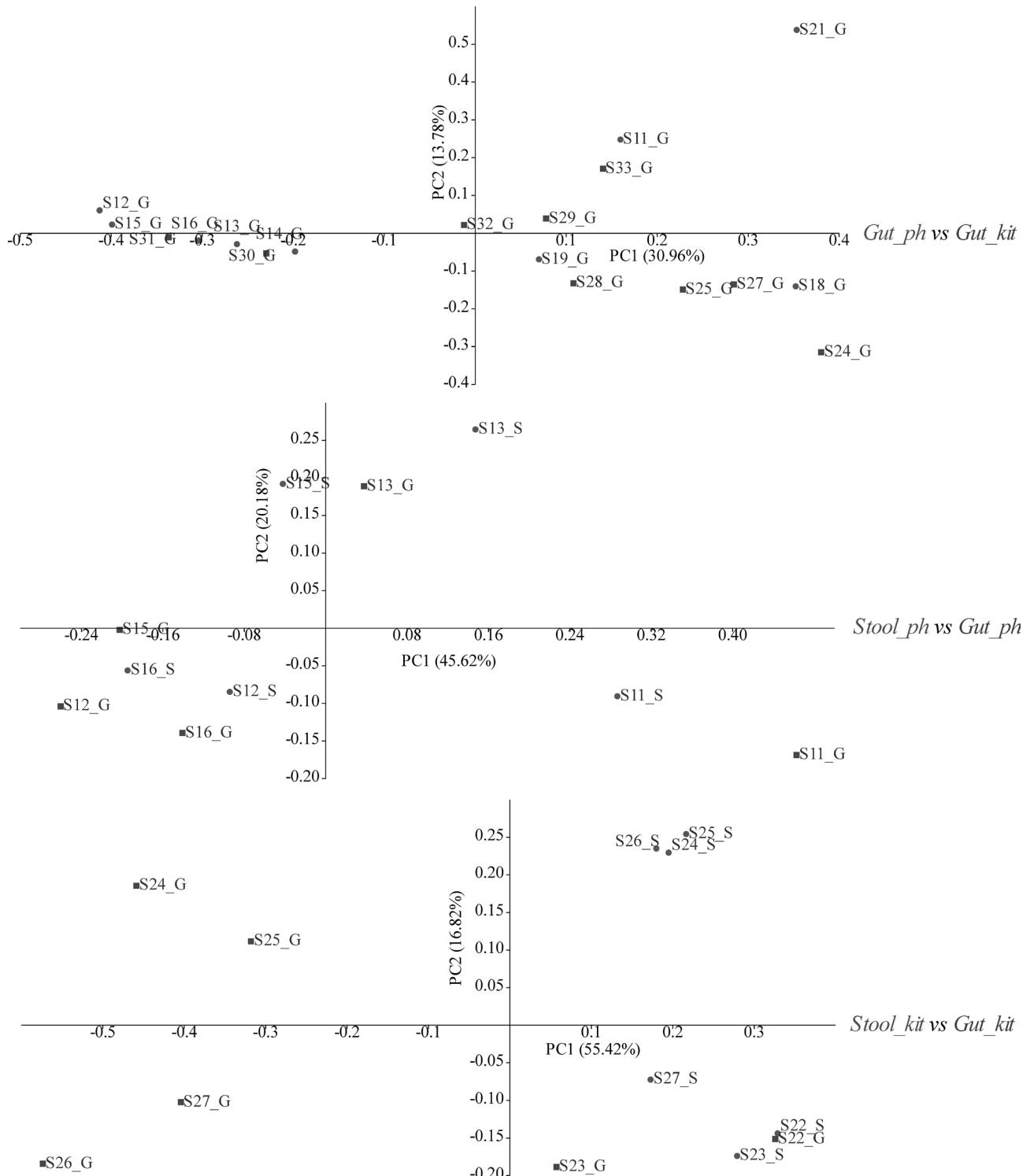


Figure 5 Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity calculated from OTU abundances in three cases, i.e., *Gut_ph vs Gut_kit*, *Stool_ph vs Gut_ph* and *Stool_kit vs Gut_kit*.

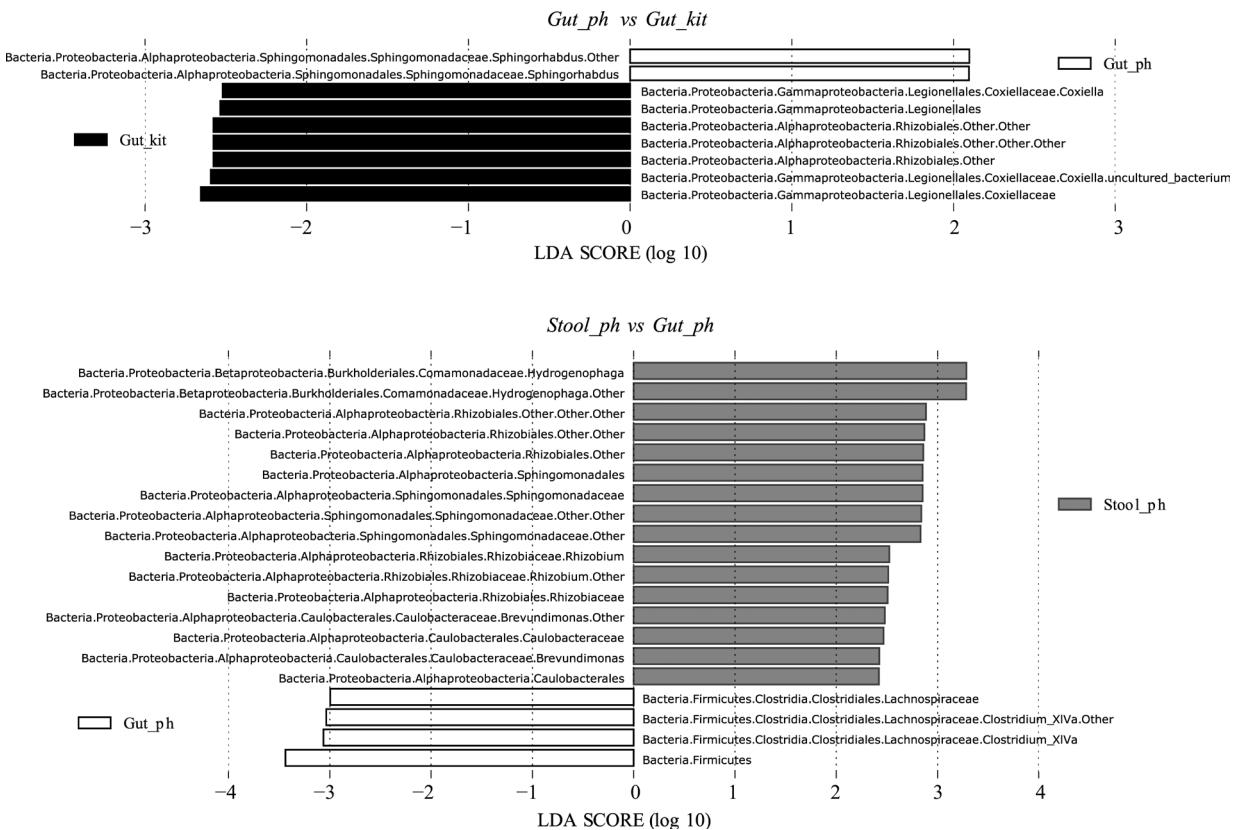


Figure 6 LEfSe results in the case of *Gut_ph* vs *Gut_kit* and *Stool_ph* vs *Gut_ph*.

genomic DNA (Ausubel, 2002). The DNA yield rate of the phenol-chloroform method is significantly higher than those of several commercial methods (Wagner Mackenzie *et al.*, 2015). Similarly, this study demonstrated the DNA extraction efficiency of the phenol-chloroform method outweighed that of TIANamp Stool DNA Kit in terms of DNA yield rate. In addition, the phenol-chloroform method produced a better A260/A230 than TIANamp Stool DNA Kit did, which probably resulted from more effective DNA washing in the last step of phenol-chloroform method. Due to the poor operability and technical overlook of mucosal bacteria in separating gut contents from intestines, we extracted the mixture of gut microbiota DNA and host genomic DNA from intestinal samples. Nevertheless, we applied the universal primer pairs to specifically amplify V3–V4 regions of bacterial 16S rDNA (Klindworth *et al.*, 2013). It has been reported that the mechanical treatments (e.g., bead beating) of samples can produce more efficient cell lysis of Gram-positive bacteria and yield high amounts of DNA (Ferrand *et al.*, 2014; Guo and Zhang, 2013). However, no method has become a gold standard for 16S rDNA high-throughput sequencing (Yamagishi *et al.*, 2016). Methods with a mechanical treatment face a tradeoff between cell

lysis efficiency and DNA disruption level. Due to the easy digestion of tadpole intestines and feces we did not take the mechanical treatments into account.

The composition and diversity of gut microbiota are undoubtedly affected by multiple factors, e.g., host genetic background, dietary profile and environmental situation (Dabrowska and Witkiewicz, 2016; Davenport, 2016; Jin *et al.*, 2017; Voreades *et al.*, 2014). The annotation on the gut microbiota structure can be susceptibility biased by the experimental protocols (Choo *et al.*, 2015; Claassen *et al.*, 2013; Yuan *et al.*, 2012). However, many studies have demonstrated that biological variations outweigh technical variations generated by DNA extraction methods (Salonen *et al.*, 2010; Wagner Mackenzie *et al.*, 2015; Wesolowska-Andersen *et al.*, 2014) and sample storage conditions (Blekhman *et al.*, 2016; Fouhy *et al.*, 2015). Here we homogenized and minimized biological variations in between groups as far as possible. Nevertheless, it is impossible to eliminate each interfering factor. For instance, the littermate tadpole pairs used in *Gut_ph* vs *Gut_kit* possess a similar but not an identical genetic background, thereby the between-method heterogeneity is possibly enhanced. From the analyses of *Gut_ph* vs *Gut_kit*, the technical variation generated

by DNA extraction methods was outweighed by the inter-subject variation. However, the phenol-chloroform method and the commercial stool kit probably resulted in a significant inconsistency in the structural composition of microbiota, e.g., OTU and phylum abundances. To ensure biological differences outweigh systematic biases, we had better use the identical standardized protocols for the comparative analysis of gut microbial consortia.

Although the microbiota structure shows a significant variation between feces and intestinal contents in the case of the sophisticated intestines (Gu *et al.*, 2013), feces has been applied as an effective noninvasive material for the study of gut microbiota in mammals. Larsen *et al.* (2015) used ribosomal intergenic spacer analysis to reveal that the microbiota structure of fishes was similar but significant different in feces and intestines. However, their preparation procedure of fecal and intestinal samples, i.e., the fecal samples were squeezed from the intestinal samples, probably enhance the between-material variations. In this study, the fecal microbiota based on the phenol-chloroform method can more efficiently reflect the gut microbiota in terms of composition and diversity. On the contrary, the feces and intestines possess more inconsistent microbiota structures deduced from the commercial stool kit. When we applied the PCoA to compare the samples fed with different food types, the experimental material factor seemed to be dominant rather than genetic background and food type factors in the case of commercial stool kit. We will explore and discuss the effects of multi-factors (e.g., genetic background and food type) on the microbiota structure of Asiatic toad tadpoles in a further study. Even though no significant difference between fecal and intestinal microbiota was detected in the phenol-chloroform method, between-material variations do exist, e.g., four genera in phylum Proteobacteria were more abundant in feces and one genus in phylum Firmicutes in intestines. We argue that the inconsistency possibly resulted from the moderate interference of microorganisms in water and tadpole skin to feces.

5. Conclusion

According to the DNA extraction quality and structural comparisons between fecal and intestinal microbiota, the phenol-chloroform method is probably more robust than a commercial fecal reagent kit (TIANGEN Biotech Co., Ltd.) in evaluating the gut microbiota structure of amphibian tadpoles with feces. To the best of our knowledge, this study provides the first evidence that

feces of amphibian tadpoles can be applied as an effective noninvasive material for the study of gut microbiota.

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Appendix

Table S1 The DNA extraction quality of samples.

Sample	Individual	Sampling date	Breed: Location	Type	Weight (mg)	DNA extraction method	A260/A280	A260/A230	DNA yield rate (ng/mg)
S1	n.a.	n.a.	n.a.	mixed stool	953.5	phenol-chloroform	1.809	1.167	5.24
S2	n.a.	n.a.	n.a.	mixed stool	467.4	phenol-chloroform	1.802	1.333	41.93
S3	n.a.	n.a.	n.a.	mixed stool	420.1	phenol-chloroform	1.881	1.487	39.51
S4	n.a.	n.a.	n.a.	mixed stool	75.7	phenol-chloroform	1.908	1.641	243.73
S5	n.a.	n.a.	n.a.	mixed stool	39.5	phenol-chloroform	1.908	1.564	430.38
S6	n.a.	n.a.	n.a.	mixed stool	60.9	phenol-chloroform	1.967	1.729	321.22
S7	n.a.	n.a.	n.a.	mixed stool	60.0	phenol-chloroform	1.627	1.204	90.00
S8	n.a.	n.a.	n.a.	mixed stool	60.7	phenol-chloroform	1.676	1.224	253.29
S9	n.a.	n.a.	n.a.	mixed stool	62.0	phenol-chloroform	1.948	1.757	210.08
S10	n.a.	n.a.	n.a.	mixed stool	60.1	phenol-chloroform	1.934	1.654	274.13
S6	n.a.	n.a.	n.a.	mixed stool	202.6	kit	1.984	1.954	24.56
S7	n.a.	n.a.	n.a.	mixed stool	208.0	kit	1.933	1.068	13.34
S8	n.a.	n.a.	n.a.	mixed stool	203.6	kit	2.010	1.544	25.54
S9	n.a.	n.a.	n.a.	mixed stool	200.0	kit	1.862	1.407	12.56
S10	n.a.	n.a.	n.a.	mixed stool	201.9	kit	2.044	1.770	10.65
S11_S	S11	20160228	I: 32°22'N, 114°19'E	stool	n.a.	phenol-chloroform	1.580	0.901	n.a.
S12_S	S12	20160228	I: 32°22'N, 114°19'E	stool	n.a.	phenol-chloroform	1.748	0.886	n.a.
S13_S	S13	20160228	II: 32°21'N, 114°18'E	stool	n.a.	phenol-chloroform	1.662	0.823	n.a.
S14_S	S14	20160228	II: 32°21'N, 114°18'E	stool	n.a.	phenol-chloroform	1.903	1.458	n.a.
S15_S	S15	20160229	III: 32°09'N, 114°02'E	stool	n.a.	phenol-chloroform	1.425	1.760	n.a.
S16_S	S16	20160229	III: 32°09'N, 114°02'E	stool	n.a.	phenol-chloroform	1.900	1.546	n.a.
S11_G	S11	20160228	I: 32°22'N, 114°19'E	gut	n.a.	phenol-chloroform	1.842	1.705	n.a.
S12_G	S12	20160228	I: 32°22'N, 114°19'E	gut	n.a.	phenol-chloroform	1.882	1.938	n.a.
S13_G	S13	20160228	II: 32°21'N, 114°18'E	gut	n.a.	phenol-chloroform	1.900	1.983	n.a.
S14_G	S14	20160228	II: 32°21'N, 114°18'E	gut	n.a.	phenol-chloroform	1.926	2.024	n.a.
S15_G	S15	20160229	III: 32°09'N, 114°02'E	gut	n.a.	phenol-chloroform	1.745	1.364	n.a.
S16_G	S16	20160229	III: 32°09'N, 114°02'E	gut	n.a.	phenol-chloroform	1.712	0.873	n.a.
S17_G	S17	20160226	IV: 32°06'N, 114°01'E	gut	28.5	phenol-chloroform	1.767	1.665	202.52
S18_G	S18	20160305	V: 32°19'N, 114°18'E	gut	61.9	phenol-chloroform	1.827	1.723	81.87
S19_G	S19	20160305	V: 32°19'N, 114°18'E	gut	46.3	phenol-chloroform	1.834	2.125	222.89
S20_G	S20	20160226	VI: 32°06'N, 114°01'E	gut	46.2	phenol-chloroform	1.888	1.728	187.01

(Continued Table S1)

Sample	Individual	Sampling date	Brood: Location	Type	Weight (mg)	DNA extraction method	A260/A280	A260/A230	DNA yield rate (ng/mg)
S21_G	S21	20160226	VI: 32°06'N, 114°01'E	gut	26.4	phenol-chloroform	1.845	2.069	488.94
S22_S	S22	20160226	IV: 32°06'N, 114°01'E	stool	35.8	kit	1.750	1.058	40.22
S23_S	S23	20160228	VII: 32°21'N, 114°18'E	stool	14.2	kit	1.531	1.588	59.15
S24_S	S24	20160305	V: 32°9'N, 114°18'E	stool	4.1	kit	1.762	0.944	266.34
S25_S	S25	20160305	V: 32°19'N, 114°18'E	stool	11	kit	1.707	0.554	240
S26_S	S26	20160226	VI: 32°06'N, 114°01'E	stool	10.3	kit	1.842	0.479	155.34
S27_S	S27	20160226	VI: 32°06'N, 114°01'E	stool	17.3	kit	2.342	0.588	37.69
S22_G	S22	20160226	IV: 32°06'N, 114°01'E	gut	22.5	kit	1.833	1.739	168.89
S23_G	S23	20160228	VII: 32°21'N, 114°18'E	gut	14	kit	1.870	1.751	126.57
S24_G	S24	20160305	V: 32°9'N, 114°18'E	gut	28.3	kit	1.837	1.000	82.4
S25_G	S25	20160305	V: 32°19'N, 114°18'E	gut	9.8	kit	1.758	0.505	248.98
S26_G	S26	20160226	VI: 32°06'N, 114°01'E	gut	18.7	kit	1.846	1.014	49.84
S27_G	S27	20160226	VI: 32°06'N, 114°01'E	gut	30.4	kit	1.758	1.415	64.47
S28_G	S28	20160228	I: 32°22'N, 114°19'E	gut	n.a.	kit	1.817	1.276	n.a.
S29_G	S29	20160228	I: 32°22'N, 114°19'E	gut	n.a.	kit	1.781	1.761	n.a.
S30_G	S30	20160228	II: 32°21'N, 114°18'E	gut	n.a.	kit	1.646	0.662	n.a.
S31_G	S31	20160228	II: 32°21'N, 114°18'E	gut	n.a.	kit	1.759	1.368	n.a.
S32_G	S32	20160229	III: 32°09'N, 114°02'E	gut	n.a.	kit	1.904	1.627	n.a.
S33_G	S33	20160229	III: 32°09'N, 114°02'E	gut	n.a.	kit	1.856	1.254	n.a.

Table S2 Two-way ANOVA on α diversity indexes.

	Source of variation	<i>df</i>	<i>F</i>	<i>P</i>
Richness	Materials	1	0.92	0.34
	Methods	1	0.83	0.37
	Materials \times Methods	1	1.86	0.18
Shannon	Materials	1	0.00	0.97
	Methods	1	0.07	0.79
	Materials \times Methods	1	0.02	0.88
Chao1	Materials	1	1.44	0.24
	Methods	1	0.84	0.37
	Materials \times Methods	1	3.85	0.06
ACE	Materials	1	1.53	0.23
	Methods	1	0.36	0.56
	Materials \times Methods	1	4.10	0.05
Simpson	Materials	1	0.09	0.77
	Methods	1	0.05	0.82
	Materials \times Methods	1	0.20	0.66
Coverage	Materials	1	1.93	0.18
	Methods	1	0.03	0.86
	Materials \times Methods	1	3.23	0.08
Shannoneven	Materials	1	0.05	0.82
	Methods	1	0.02	0.90
	Materials \times Methods	1	0.16	0.69

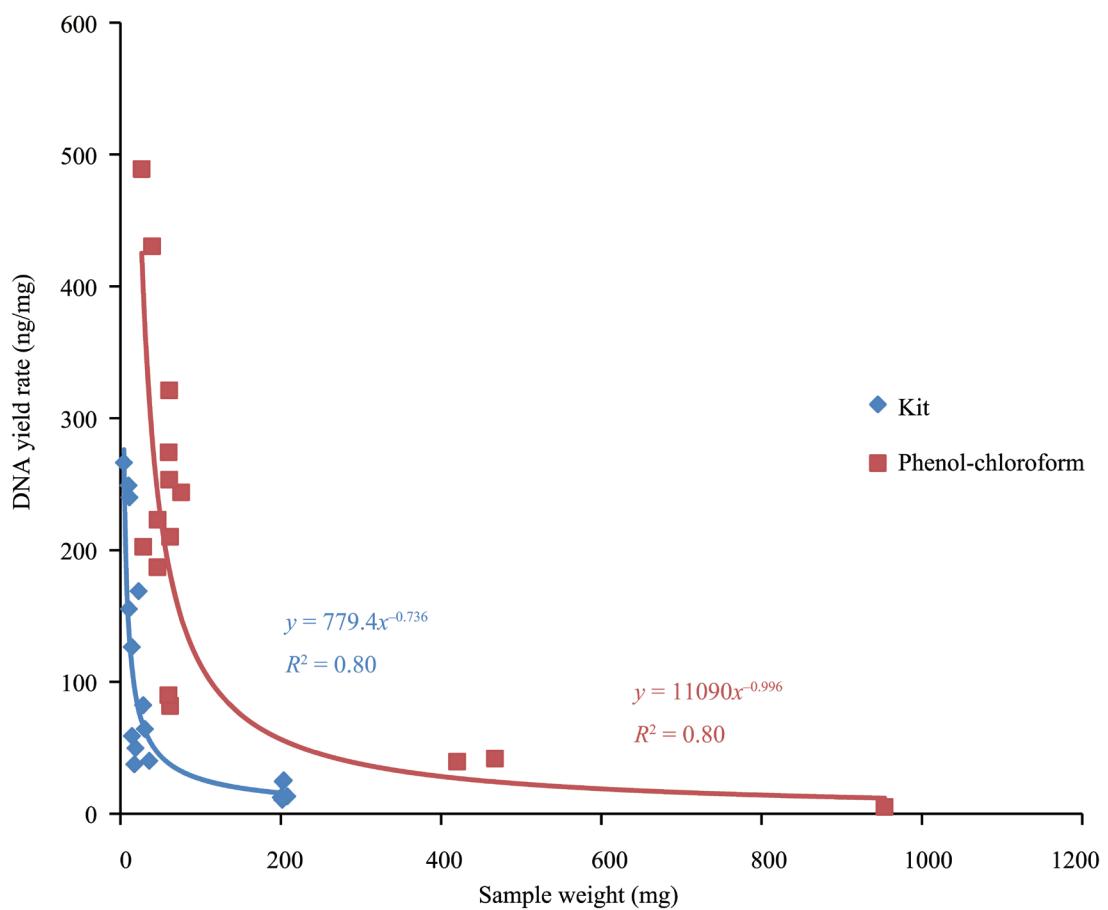


Figure S1 Relationship between DNA yield rate and sample weight in TIANamp Stool DNA Kit ($n = 17$) and phenol-chloroform method ($n = 15$).

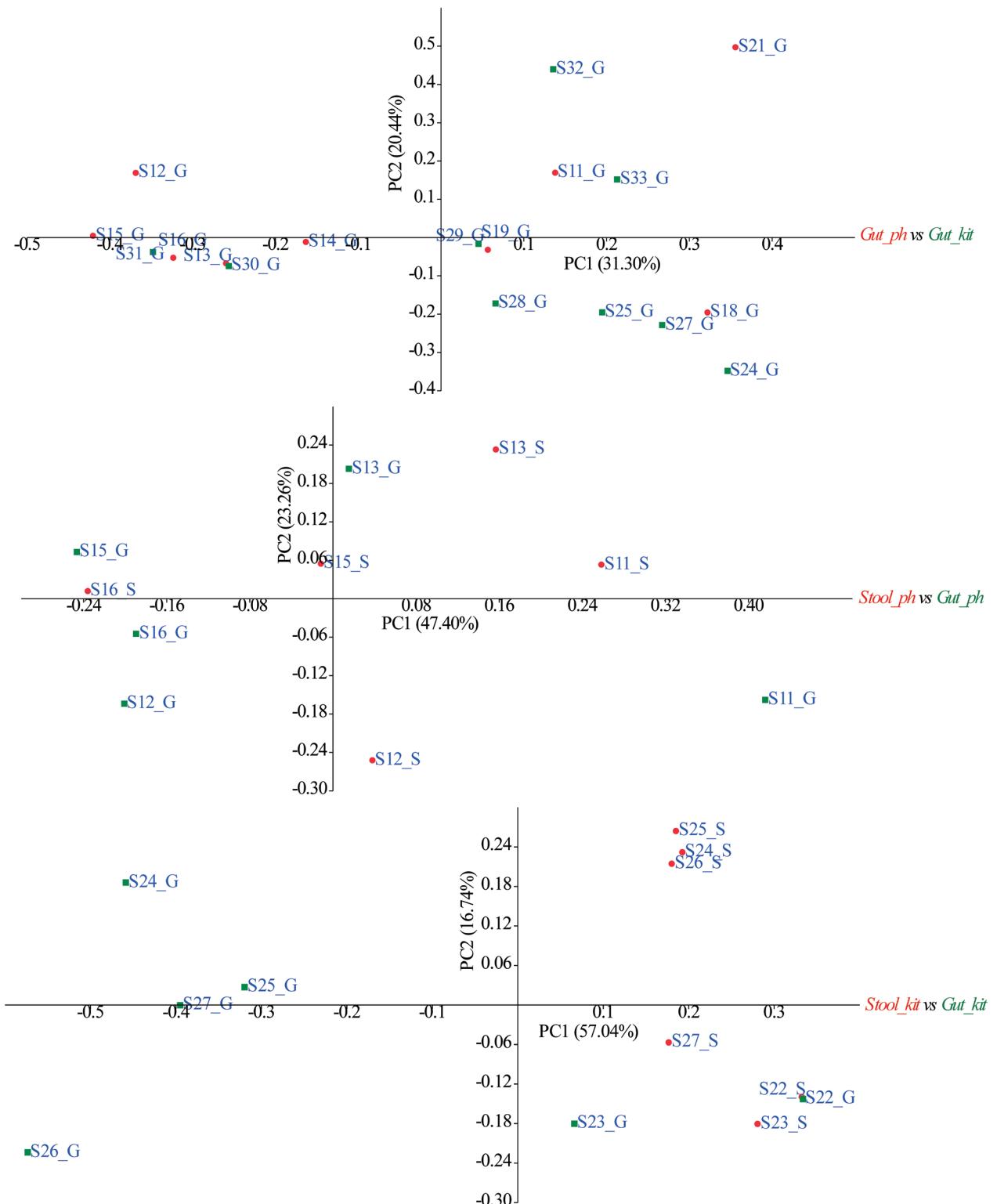


Figure S2 PCoA on Bray-Curtis dissimilarity calculated from genus abundances in three cases, i.e., *Gut_ph* vs *Gut_kit*, *Stool_ph* vs *Gut_ph* and *Stool_kit* vs *Gut_kit*.

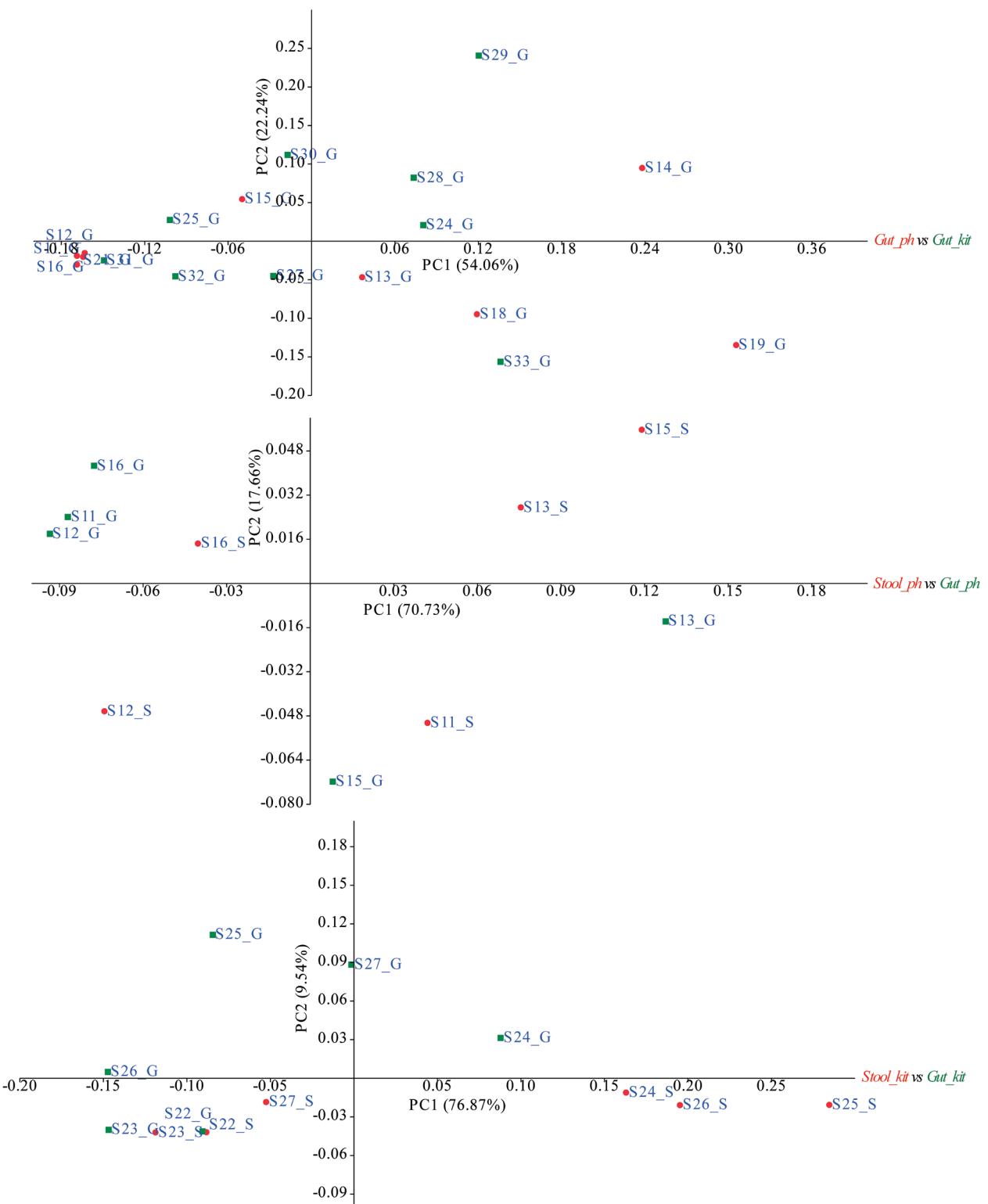


Figure S3 PCoA on Bray-Curtis dissimilarity calculated from phylum abundances in three cases, i.e., *Gut_ph* vs *Gut_kit*, *Stool_ph* vs *Gut_ph* and *Stool_kit* vs *Gut_kit*.

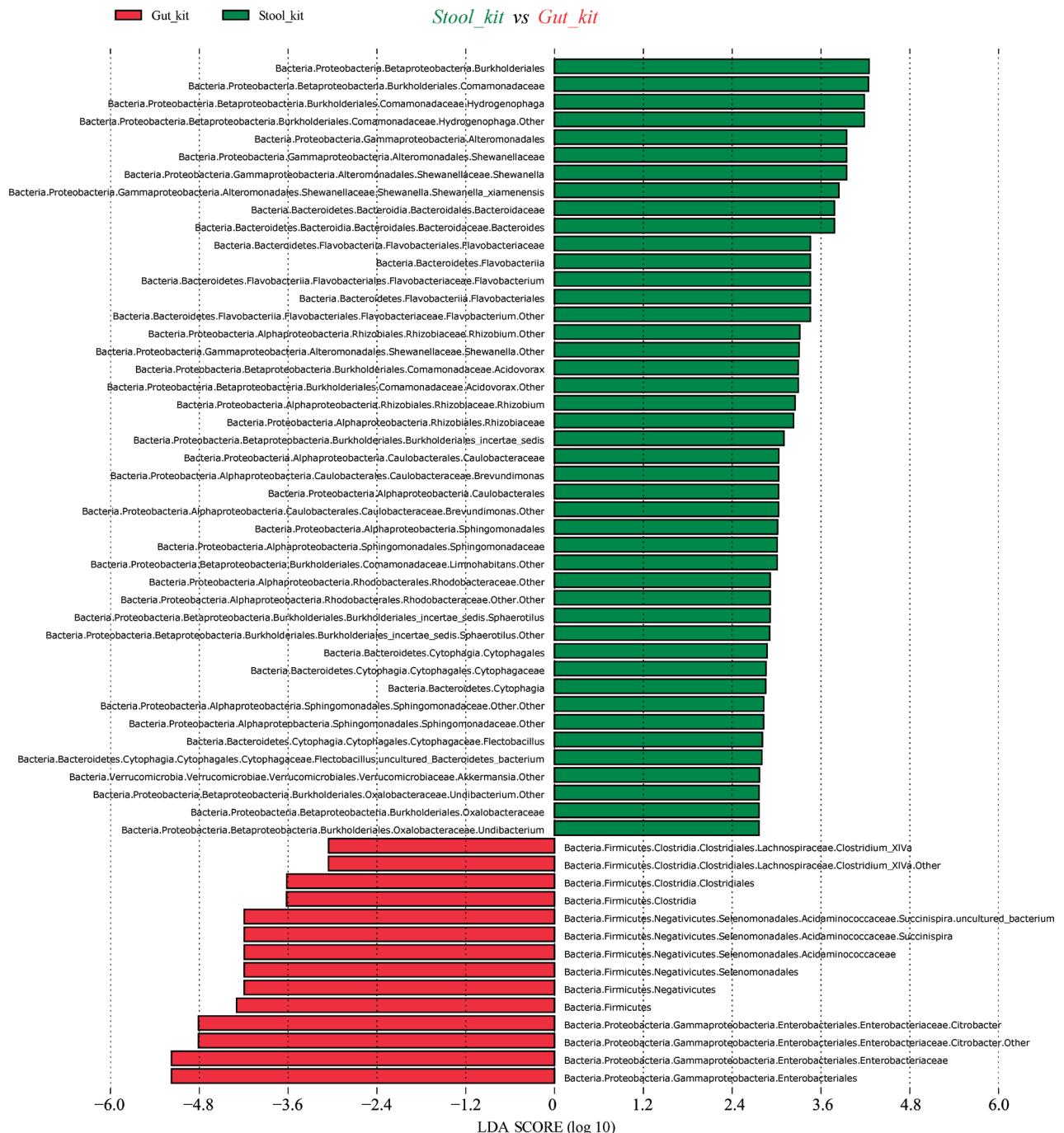


Figure S4 LEfSe results in the case of *Stool_kit* vs *Gut_kit*.